ON THE THERMODYNAMICS AND KINETICS OF THE COOPERATIVE BINDING OF BACTERIOPHAGE T4-CODED GENE 32 (HELIX DESTABILIZING) PROTEIN TO NUCLEIC ACID LATTICES

Stephen C. Kowalczykowski, Nils Lonberg, John W. Newport, Leland S. Paul, and Peter H. von Hippel, *Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403 U.S.A.*

ABSTRACT In this paper we summarize a series of thermodynamic, and preliminary kinetic, studies on the molecular details and specificity of interaction of phage T4-coded gene 32-protein (GP32) with nucleic acid lattices. It is shown that the binding of GP32 to short (l = 2–8 residues) oligonucleotides is essentially independent of base composition and sugar-type, as well as of salt concentration. In contrast, cooperative (continuous) or isolated binding of GP32 to single-stranded polynucleotides is base and sugar composition-dependent (binding is tighter to DNA than to RNA) and highly dependent on salt concentrations. Binding constants (K), cooperativity parameters (ω), and binding site sizes (n) are determined for binding to various nucleic acid lattices under a variety of environmental conditions. These results are used to show that GP32 can bind to nucleic acid lattices in two different conformations, and to characterize the molecular details of these binding species. Further insight into the molecular origins of binding cooperativity is obtained by determining these thermodynamic parameters also for the specifically proteolytically degraded GP32 fragments GP32* I (C-terminal peptide removed) and GP32* III (C- and N-terminal peptides removed). It is also shown that these GP32-nucleic acid binding measurements can be used to provide a quantitative molecular interpretation of the sequential (competitive) binding equilibria involved in the autogenous translational regulation of GP32 synthesis (Lemaire et al., 1978, *J. Mol. Biol.* **126**:73, 1978), and to illustrate some general principles of the development of interactional specificity in cooperatively binding protein-nucleic acid complexes. Preliminary experiments have also been carried out on the kinetics of GP32 association to, and dissociation from, single-stranded nucleic acid lattices. In particular, fluorescence stopped-flow measurements of the dissociation of GP32 from such lattices as a function of lattice saturation (and protein cluster size) can be interpreted to suggest that the protein may translocate ("slide") on the lattice before dissociation. These studies permit an approach to possible rates and mechanisms of such translocation events.

INTRODUCTION

Bacteriophage T4-coded gene 32-protein (GP32) binds preferentially and cooperatively to single-stranded nucleic acids, and has been shown by genetic and biochemical analysis to be an essential component of the DNA replication, recombination, and repair processes involved in the development of the phage (for review, see reference 1). The biological activity of GP32

Dr. Lonberg's present address is Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Mass. 02138.

Dr. Newport's present address is Department of Biochemistry and Biophysics, University of California, San Francisco, Calif. 94143.

appears to reside in its binding properties; it is thought to function by binding to and stabilizing (as well as protecting against nucleases) the transient single-stranded intermediate structures involved in the above "DNA-manipulation" processes.

The thermodynamics of the interaction of GP32 (and specifically proteolytically degraded GP32 derivatives) with nucleic acids have been described in detail elsewhere (2–5, footnotes 1–3). In this paper we will review and summarize these studies, stressing the molecular structures and competitive binding equilibria involved in the function of this protein, and attempt to formulate some general principles underlying the specificity of interaction of cooperatively binding genome regulatory proteins with nucleic acids.

In addition, since DNA replication forks and recombinational intermediates represent transient (time-dependent) structures in the ongoing processes of DNA synthesis and manipulation, we present here some preliminary measurements of the rates of GP32 association to, and dissociation from, single-stranded polynucleotides. These findings, together with those of Lohman (6), suggest that in addition to direct association and dissociation reactions, GP32 is capable of limited one-dimensional translocation ("sliding") along nucleic acid lattices.

MATERIALS AND METHODS

Details of protein purification, characterization of nucleic acid lattices, and experimental procedures have been presented elsewhere (3-5; footnotes 1–3). Fluorescence titrations and spectra were carried out using either a Varian 620i computer (Varian Associates, Palo Alto, Calif.), controlled spectrofluorimeter (Schoeffel Instrument Corp., Westwood, N.J.) or a Hitachi MPF-2A spectrofluorimeter (Hitachi Limited, Tokyo, Japan). UV titrations were done on a Varian 620i-controlled Cary 14 spectrophotometer (Cary Instruments, Monrovia, Calif.), and theoretical modeling studies were performed either on a Varian 620i or on a PDP-10 computer (Digital Equipment Corp., Maynard, Mass.). Fluorescence stopped-flow experiments were conducted on a modified Durrum instrument (Durram Instrument Corp., Sunnyvale, Calif.) also interfaced to a Varian 620i. All experiments were conducted at 25 ± 1°C, unless otherwise indicated.

RESULTS AND DISCUSSION

Thermodynamic Aspects

Initial Measurements Alberts and co-workers (2, 7) first isolated and purified GP32, and demonstrated in vitro that its central functional feature is its ability to bind preferentially and cooperatively to single-strand DNA sequences. Subsequent studies from this laboratory (3–5) initiated a quantitative thermodynamic examination of this property of the protein. It was shown that GP32 can bind to short (l = 2–8 residues) oligonucleotides with an apparent association constant of $\sim 10^5\ M^{-1}$, and that this binding appeared to be essentially independent of oligonucleotide base composition and sugar type. In addition, measurements of the cooperative binding of GP32 to polynucleotide lattices revealed the binding site size of the protein (n) to be ~7 nucleotide residues, the binding constant (K) to be $\sim 10^4$–$10^6\ M^{-1}$ in 0.1 M NaCl, and the cooperativity parameter (ω) to be $\sim 10^3$. (See reference 8 for definitions and descriptions of these binding parameters.) Even though GP32 is thermodynamically defined as a "melting protein" as a consequence of its preferential binding to single-stranded nucleic

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[1]Kowalczykowski, S. C., N. Lonberg, J. W. Newport, and P. H. von Hippel. Manuscript submitted for publication.

[2]Newport, J. W., N. Lonberg, S. C. Kowalczykowski, and P. H. von Hippel. Manuscript submitted for publication.

[3]Lonberg, N., S. C. Kowalczykowski, L. S. Paul, and P. H. von Hippel. Manuscript submitted for publication.

acid sequences, it has been shown (2, 3) that this protein is "kinetically blocked" from actually melting native DNA (though it can melt double-stranded poly d[A-T] to equilibrium).

In Vivo Titration of Single-Stranded DNA Sequences and Autogenous Regulation of GP32 Synthesis Concurrently with the above studies, Gold and co-workers (9, 10) and Krisch et al. (11) demonstrated in vivo that the amount of GP32 synthesized in a T4 infection is proportional to the amount of single-stranded DNA present. Subsequently, Lemaire et al. (12) showed, using an in vitro translation system, that GP32 synthesis is autogenously regulated (see Fig. 4, below). They found that after all of the single-stranded DNA sequences present in the solution had been complexed with GP32, the free concentration of the protein increases to a critical level. At this point the protein binds specifically to its own (homologous) mRNA, shutting off further synthesis of GP32 without interfering with the synthesis of other T4 proteins. These findings, demonstrating binding specificity to various nucleic acid substrates in vivo, seemed incompatible with our earlier demonstration of apparent nonspecificity of binding at the oligonucleotide level, and led us to a further examination of the binding specificity of GP32.

Oligonucleotide Titrations To this end we[1] repeated and extended the earlier oligonucleotide titrations of Kelly et al. (5), monitoring quenching of the intrinsic (tryptophan) GP32 fluorescence on oligonucleotide binding. Working with higher precision techniques we showed unequivocally (within a factor of ~3 in K) that the binding (K_{oligo}) of GP32 to oligonucleotides 2–8 residues long is independent of base composition and oligonucleotide length, and that binding to RNA oligonucleotides is at most threefold weaker than binding to DNA oligonucleotides of the same length and composition. In addition, we showed that K_{oligo} for GP32 binding to oligonucleotides is approximately independent of salt concentration ($\partial \log K_{oligo}/\partial \log [NaCl] \simeq -0.3$; see reference 13 for general treatment and interpretation of such data). The lack of specificity in oligonucleotide binding seemed indeed to suggest that the demonstrated physiological binding specificity must reflect either an enhancement of small differences in affinity as a consequence of protein monomer binding in cooperative clusters (14), or that binding to polynucleotide lattices must involve different GP32-nucleic acid interactions (or, of course, both of the above).

Polynucleotide Titrations To investigate this aspect we undertook a comprehensive series of titrations of various homopolynucleotides with GP32. Titrations were carried out by monitoring either the quenching of intrinsic protein fluorescence on binding, or the UV absorbance change of the nucleic acid due to the base unstacking and nucleic acid lattice conformational change that accompanies GP32 binding (3). Titrations were run as a function of salt concentration, and typical results (here with poly rA) at different salt concentrations are presented in Fig. 1. Clearly, binding is cooperative and salt-concentration-dependent.

The free ligand (GP32) concentration at the midpoint of titrations such as those of Fig. 1 is equal to $(K\omega)^{-1}$ for that polynucleotide at that salt concentration. Values of $K\omega$ measured in this way on a variety of polynucleotides are presented as a plot of log $K\omega$ versus log [NaCl] in Fig. 2. This figure clearly shows that the effective binding constant ($K\omega$) for GP32 (at constant salt concentration) is dependent on both the base composition and the sugar type of the polynucleotide. Quantitative analysis of data such as that of Fig. 2 shows that $K\omega$ for a random copolymer containing several types of bases (or for a natural DNA) is approximately equal to the compositionally-weighted sum of the $K\omega$ values for the individual homopolynucleotides, indicating that specificity depends on differential binding of individual bases along the chain. In addition, $K\omega$ for a given polydeoxyribonucleotide is always greater than that for the homologous polyribonucleotide (Fig. 2).

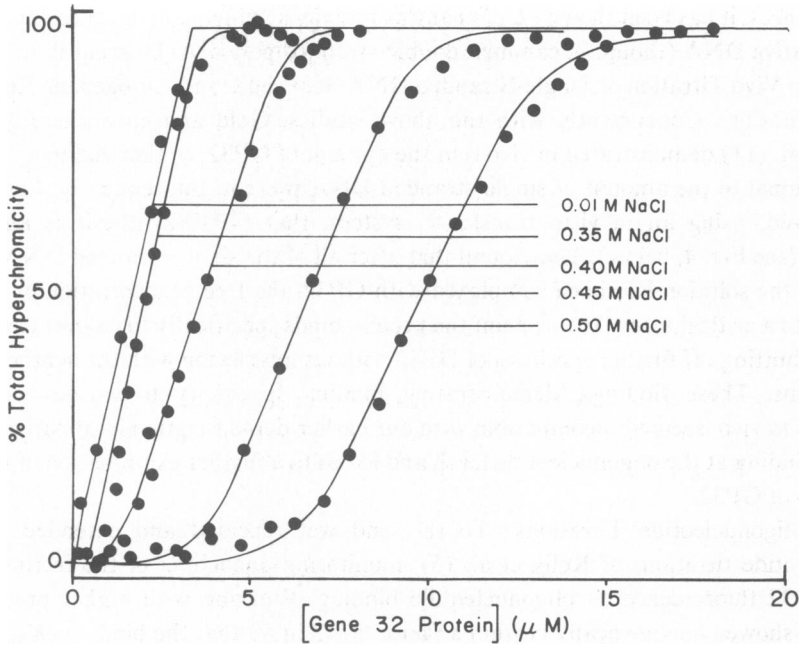

Figure 1 Salt concentration dependence of the binding of GP32 to poly rA in 10 mM Hepes, 0.1 mM EDTA, pH 7.7, plus added NaCl as indicated. The solid lines represent calculated theoretical curves, using the following parameters: $n = 7$ nucleotide residues/GP32 monomer, $\omega = 2 \times 10^3$, and K was determined (using this ω) from the experimental values of $K\omega$.

Both Figs. 1 and 2 also show that, unlike oligonucleotide binding, cooperative polynucleotide binding is very salt-concentration dependent ($\partial \log K\omega / \partial \log [\mathrm{NaCl}] \simeq -6 \pm 1$). In addition, binding measurements performed with mono- and divalent salts carrying different anions suggest that approximately two-thirds of the above salt dependence reflects displacement of protein-bound anions as a consequence of nucleic acid interactions. Studies involving

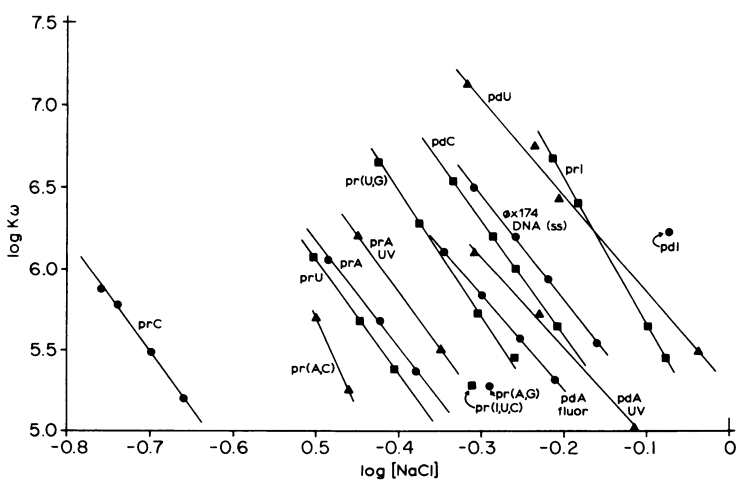

Figure 2 Plot of log $K\omega$ versus log [NaCl] for the cooperative binding of GP32 to various polynucleotides, as indicated in the figure; buffer as in Fig. 1. Lines marked fluor or UV represent duplicate sets of measurements carried out by quenching of intrinsic protein fluorescence, or changes in UV absorbance, respectively. The rest of the data were all determined by the fluorescence quenching methods.

the fit of a theoretical binding equation (8) to experimental titration data, and measurements at very low binding densities (ν), show that all the salt dependence of $K\omega$ is in K, with ω remaining constant (salt-independent) at $\sim 10^3$.

These results indicate that GP32 can bind to nucleic acids in two very different ways, probably representing two distinct protein conformations. We term these binding conformations the oligonucleotide and the polynucleotide binding modes, respectively. Fig. 3 illustrates and summarizes some of the inferred molecular features of these two binding modes in schematic form (for further details see footnote 1).

Binding Properties of GP32* I and GP32* III Hosoda and co-workers (15, 16) have shown that brief treatment of native GP32 with proteolytic enzymes results in cleavage of an ~60 residue peptide from the C-terminus, and an ~20 residue peptide from the N-terminus of the original protein. These cleavage products can be isolated from such a digest; the resulting proteins have been termed GP32* I (C-terminal peptide removed), GP32* II (N-terminal peptide removed), and GP32* III (both peptides removed). These cleaved proteins show changes in both apparent binding affinity and cooperativity of binding to nucleic acid lattices, relative to undegraded GP32 (15–17). To further our understanding of the molecular interactions responsible for GP32 complex formation with nucleic acids, we have measured the thermodynamic parameters characterizing the binding of GP32* I and GP32* III to nucleic acid lattices.[3]

GP32* I binds to short oligonucleotides with approximately the same K as GP32 (at ~0.1 M NaCl), but with a somewhat increased salt dependence. Binding of GP32* I to the various polynucleotides of Fig. 2 follows the same order of binding affinity, and shows the same overall salt dependence on GP32; n and ω are unchanged from the GP32 values, and K is

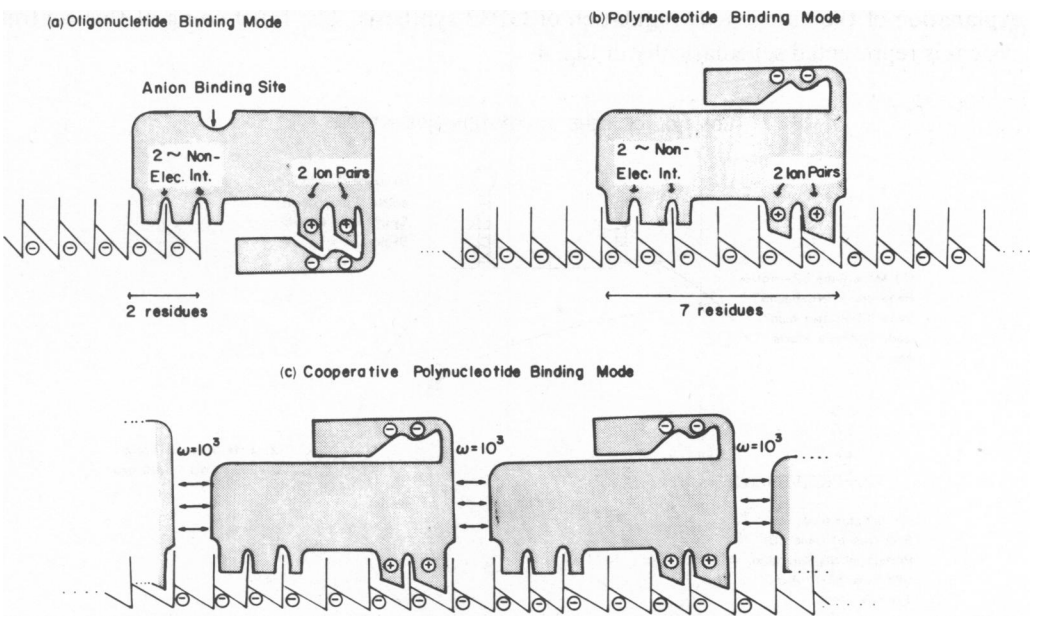

Figure 3 Schematic representation of the present information about the binding modes (conformations) of GP32 to nucleic acid lattices. Note that binding in the polynucleotide mode involves unmasking of a largely electrostatically-binding subsite, with the concomitant removal of the block to statistical "shuffling" binding seen in the oligonucleotide mode, and also the disruption of the "anion binding site." Cooperative binding in the polynucleotide mode involves lattice distortion and protein-protein interaction (see text and, for further details, footnote 1).

increased ~ two- to fourfold. These results are all consistent with a partial proteolytic removal of the negatively charged "shuffling block" in GP32 (Fig. 3). GP32* I thus appears to be very similar thermodynamically to GP32. The small difference in K we have determined does not appear to be adequate to account for the fact that this derivative of GP32 can melt native DNA to equilibrium (see reference 15 and kinetics discussion below).

On the other hand, GP32* III is a very different protein from native GP32. In particular, GP32* III shows no trace of cooperativity in its interactions with polynucleotides; i.e., $\omega = 1$. Therefore, its net binding affinity is much lower, with values of K ranging from $\sim 10^6$ M^{-1} at 0.05 M NaCl to $\sim 10^4$ M^{-1} at 0.3 M NaCl for poly rA and poly dA. There is also a much smaller dependence of K on salt, with $\partial \log K/\partial \log [NaCl] = -2.8$ for poly dA. Some base and sugar specificity is present, but not of the magnitude observed for GP32 and GP32* I. Thus it appears that removal of only 20 amino acid residues from the amino terminus of GP32 results in a totally different protein, though we have observed that both GP32* I and GP*III (as well as GP32) unstack the bases and deform the backbone on binding to a DNA lattice. This suggests (3, 4, footnote 1) that lattice deformation alone is not responsible for protein binding cooperativity; direct protein-protein interactions must also be involved.

Competitive Cooperative "NonSpecific" Binding as a Genome Regulatory Mechanism As described above, gene 32-protein binding in the polynucleotide mode does indeed exhibit differences in net affinity ($K\omega$) for different nucleic acid lattices at constant salt concentration (Fig. 2), depending on base composition and sugar type. In addition, at higher salt concentrations (such as those characterizing the in vitro translation system of Lemaire et al. [12]) we see a finite "lag phase" in the cooperative titration curve (e.g., see Fig. 1), before the onset of lattice binding. That is, the free protein concentration must reach a certain critical level before binding begins. These properties can be utilized to provide a quantitative explanation of the autogenous regulation of GP32 synthesis. The functioning of this control system is represented schematically in Fig. 4.

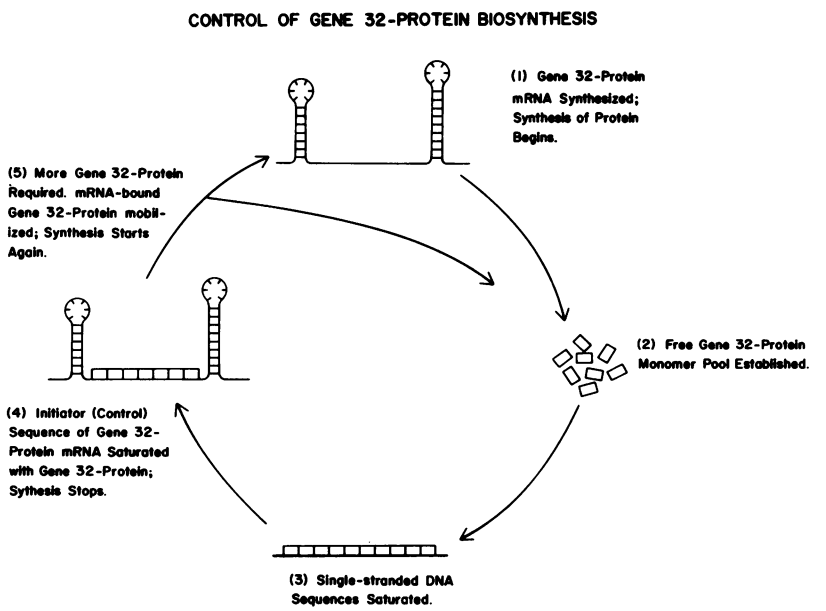

Figure 4 Schematic representation of the sequence of events involved in the autogenous regulation of GP32 synthesis, as elucidated by Gold and co-workers (10,12). The various competitive binding equilibria involved are indicated (see text).

How does such a system work? Because binding is cooperative (Fig. 1) and competitive for protein (i.e., generally there is an excess of lattice binding sites available), control depends on which of these sets of competing binding sites are saturated first. Fig. 2 suggests that for lattices of comparable size and base composition, binding to DNA sequences should precede RNA binding. (In addition [data not shown in Fig. 2] binding of GP32 to poly dT is anomalously tight, further favoring the initial saturation of DNA lattices.) Cooperativity of binding can amplify small differences in intrinsic monomer binding affinity (K) or cooperativity (ω); see reference 14. Differences in lattice size can also modulate the competition for binding ligand because binding is cooperative; this means that given two competing lattices of identical base composition and sugar type, the larger lattice will saturate first. Thus, as originally suggested by Russel et al. (9), gene 32 mRNA may carry a critical control sequence (flanked, for example, by two stable hairpins) which is larger than such sequences on other T4 mRNAs, but is effectively shorter than the average single-strand DNA sequence present during replication. Such models could clearly also account for the sequential regulation processes indicated in Fig. 4. Elsewhere[2] we have modeled such possible schemes, and have shown not only that such systems be made quantitatively consistent with the gene 32-protein autoregulation data (12), but also that these approaches can be used to construct a variety of genome regulatory systems based on the general principle of specificity of control arising from the cooperative binding to competing nucleic acid sequences of proteins which are relatively "nonspecific" in their intrinsic monomer binding affinities.

Kinetic Aspects

Kinetics of GP32 Binding in DNA Melting and Replication The kinetics of the interaction of GP32 with nucleic acid lattices are of interest from several perspectives. First, as indicated above, GP32 cannot melt native DNA to equilibrium, while GP32*I can. The trivial differences in thermodynamic parameters found for these two proteins suggest that GP32*I must attack double-stranded DNA via a different kinetic pathway than GP32. Studies of this system are underway in our laboratory which we hope will illuminate some aspects of DNA "breathing," but, as a necessary prelude to such a study, the kinetics of GP32 (and GP32* I) binding to single-stranded DNA lattices must be understood.

In addition, the kinetics of binding of GP32 to single-stranded DNA may be involved in replication. Single-stranded DNA is formed at each replication fork during T4 DNA synthesis at an estimated rate of ~1,000 nucleotides/s (1). If GP32 is to saturate these single-stranded sequences as they are formed, we must ask whether the kinetics of GP32 binding to, and dissociating from, the lattice are fast enough to keep pace with the replication process, or whether the active participation of other proteins must be invoked to facilitate the association-dissociation process. This means we must consider binding pathways, asking both how a GP32 monomer "finds" and binds to a cooperatively bound cluster of GP32 molecules, and also how the dissociation of protein monomers from contiguously-bound clusters proceeds.

Binding could, in principle, involve direct random lattice association-dissociation events until, by trial and error, the protein has managed to find a cluster-contiguous binding site. Dissociation could then proceed directly from the contiguous sites. A quick calculation suggests that such a (dissociation) process might be very slow. The net binding affinity for cooperatively bound GP32 ($K\omega$) under physiological conditions is at least 10^8 M^{-1}. If association of GP32 to lattice sites is diffusion-controlled, k_a could be as large as 10^8 M^{-1} s^{-1}. The resulting dissociation ($k_d \simeq 1$ s^{-1}) would be much too slow to keep pace with the replication process.

Sliding Mechanisms A kinetic mechanism which could facilitate the achievement of binding equilibrium is a one-dimensional random walk or diffusion ("sliding") process of the type which has been suggested theoretically (18, 19) and demonstrated experimentally (20) to be involved in the location by *E. coli lac* repressor of its target DNA sequence. In this system the protein is thought to move along the DNA in an electrostatically-stabilized one-dimensional random walk, in addition to undergoing microscopic association-dissociation events into and out of solution. If the rate of sliding exceeds that of dissociation, this process can greatly facilitate the attainment of binding equilibrium.

Recently Epstein (21) has also considered the effects of such sliding on the rates of interaction of large ligands with nucleic acids, stressing the association process. Here we map out a preliminary approach (based in part on the calculations of Epstein) to the application of this concept to the kinetics of GP32-lattice complex dissociation.

Model Considerations GP32 is distributed over a partly saturated nucleic acid lattice in three thermodynamically distinct classes of binding sites (8). These are: isolated sites; singly-contiguous sites (located at the ends of a bound cluster of proteins); and doubly-contiguous sites (located in the "interior" of a bound cluster). The binding affinity of a GP32 molecule bound in these ways is K, $K\omega$ and $K\omega^2$, respectively. The time-course of the overall dissociation process should reflect the different amplitudes and rates of association of each species of ligand.

Two limiting cases will be considered. To simplify the discussion and illustrate conceptual differences, a diffusion-controlled association process with neither intermediate species nor competing pathways will be assumed. Thus all kinetic differences due to cooperative interactions will be manifest in the dissociation process.

The "No Sliding" Case This scheme assumes that the three species of bound ligand are not mobile, and therefore cannot interconvert on the lattice during the time-course of the dissociation. (However the species are not completely independent of one another since the dissociation of a doubly contiguous ligand results in the creation of two singly contiguous species; isolated ligands, on the other hand, are independent of the contiguously bound species.) The initial distribution of bound ligands will depend on n, K, and ω, which are fixed physical parameters of the protein-nucleic acid system being studied, and on initial lattice binding density (ν).

If a perturbation resulting in a net dissociation of ligand is applied to the system, the observed time-course will be determined by the initial value of ν (and, of course, n, K and ω). In general, at least initially, one should observe as many as three distinct kinetic phases, separated in dissociation constant by factors of ω and ω^2 and corresponding to dissociation of isolated, singly contiguous, and doubly contiguous ligands, respectively. The amplitudes of each phase will then depend on the relative concentrations of each species. In practice, for a ligand characterized by a large value of ω ($\sim 10^3$ for GP32), only one or two components will be detected, because one or two species will be dominant in the initial equilibrium. For example, at high values of ν the amplitude of the component due to isolated sites will be so low as to preclude detection; at low values of ν, where the concentrations of isolated and singly contiguous ligands are approximately equal, one should observe two exponential decays characterized by dissociation rates of k_d and k_d/ω, respectively. Furthermore, since each dissociation of a singly contiguous ligand from the end of a large cluster of bound proteins creates a "new" singly-contiguous ligand, we would expect relatively little change in the concentration of singly-contiguous ligands early in the dissociation; i.e., only the lengths of the clusters are decreased. This predicts that, at least initially, the rate of dissociation should be

approximately constant with time, and not first order as expected for (independent) dissociation of isolated ligands. Therefore, the observation of distinct kinetic phases would be required to demonstrate the absence of sliding, and thus the applicability of this first (oversimplified) kinetic mechanism.

The "Fast Sliding" Case If the ligands are allowed to slide rapidly along the lattice after each dissociation event, so that lattice equilibrium is maintained throughout the time-course of the reaction, the analytical expressions developed by Epstein (21) can be utilized. Fig. 5 represents the time-course of dissociation for a cooperatively interacting system ($\omega \simeq 10^3$), starting from differing values of initial fractional saturation. The most outstanding features of these curves are that the dissociation curves are distinctly sigmoid at high values of fractional saturation, and the apparent rate constant increases with time for each curve. This follows because, at the start of dissociation, the predominant population of bound ligand is either the singly or doubly contiguous species characterized by dissociation rate constants of k_d/ω and k_d/ω^2, respectively. As the reaction progresses, and new lattice equilibria are established at lower values of ν, dissociation from isolated species begins to dominate, due to their larger dissociation rate constant (k_d). Thus the apparent dissociation rate constant increases as a function of time.

If one considers the value of the initial rate constant only as a function of fractional saturation, the following expression is obtained:

$$k_{app} \simeq k_d[g(0) + \omega_d g(1) + \omega_d^2 g(2)], \quad (1)$$

where $g(0)$, $g(1)$ and $g(2)$ represent the relative probabilities of occurrence of the isolated, singly-, and doubly-contiguously bound species, respectively; and ω_d is the kinetic contribution of the cooperativity parameter to the dissociation rate constant. This expression shows that the apparent initial rate constant would be expected to vary in magnitude by ω_d^2 between 0 and 100% fractional saturation if this mechanism is followed.

Dissociation Rate Measurements Preliminary studies of the kinetics of association and dissociation of this protein from nucleic acid lattices have been undertaken by monitoring, in a stopped-flow apparatus, the changes in intrinsic protein fluorescence which accompany binding, to determine whether either of the two simple models discussed above are applicable to the GP32 system. Peterman and Wu (22) also used this technique to examine the dissociation of GP32 from fd DNA, using a "salt-jump" procedure to remove the protein. Also, Lohman (6) has carried out kinetic studies of this system. We (and Lohman) have used synthetic homopolynucleotides in these studies to avoid potential problems associated with base compositional heterogeneity and possible hairpin loop formation.

In contrast to the results with fd DNA (22), our data for the salt-jump-induced dissociation of GP32 from homopolynucleotides can be fit by a single exponential over essentially the entire time-course of the reaction. We have never observed more than one phase for the dissociation, even under low ν-conditions where up to 50% of the bound molecules exist in the isolated state. Thus these data seem incompatible with the simple nonsliding model, for which multiphasic dissociation curves would be expected.

In Fig. 6, we plot the dependence of the observed first-order rate constant for the dissociation of GP32 from poly rA against the initial fractional saturation. It is apparent that there is a distinct dependence of k_d on the initial binding density. Although the fast-sliding model predicts a strong dependence of k_d on ν, the magnitude of the dependence of the experimental data on this variable is not sufficiently great to be compatible with the

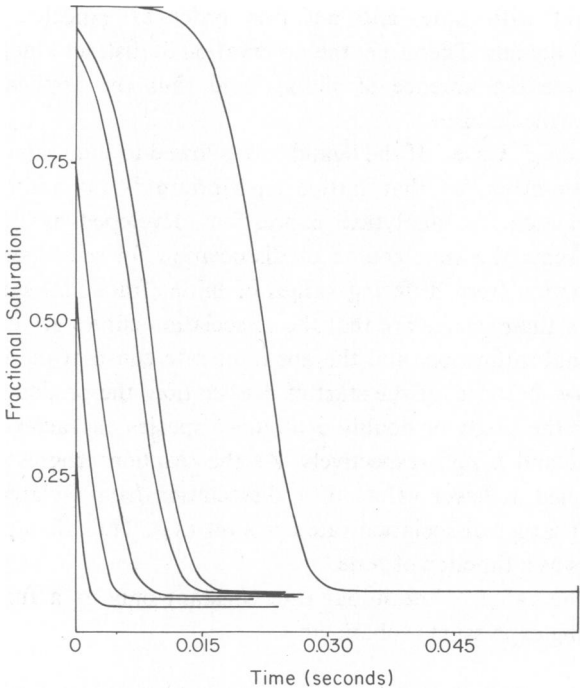

Figure 5 The course of dissociation for a cooperatively binding ligand assuming rapid sliding. The curves were generated by numerical integration of Eq. 19 in reference (21). The parameters used were: $n = 7$, $\omega = 1 \times 10^3$, $\omega_a = 1$, $\omega_d = 1 \times 10^{-3}$, $k_a = 5 \times 10^6$ and $k_d = 5 \times 10^3$. The lattice concentration was varied so that the initial values of fractional saturation are those indicated at $t = 0$.

expectations of Eq. 1; i.e., k_d at 50% saturation should be $\sim 10^3$ smaller than k_d at 0.1% saturation; this is not the case.

In Fig. 7 the salt dependence of k_d for poly dA and poly rA, at different values of initial saturation, is presented in the form of a log k_d versus log [NaCl] plot. The average slope ($\partial \log k_d / \partial \log$ [NaCl]) is 4.1 ± 0.4 for the poly rA data and is 3.0 ± 0.5 for the poly dA data. The comparable equilibrium slopes ($\partial \log K\omega / \partial \log$ [NaCl]) are −6.8 ± 0.5 for poly rA and −5.8 ± 0.5 for poly dA (Fig. 2). Thus the entire salt dependence of the equilibrium constant does not seem to appear in the dissociation rate constant.

Comparison of the dissociation rate constants for poly dA and poly rA at equivalent values of fractional saturation show that k_d is greater for the latter; e.g., k_d is ~tenfold greater for poly rA in 0.5 M NaCl. The equilibrium binding affinities for the two polynucleotides differ to about the same extent; e.g., in 0.5 M NaCl $K\omega$ is ~eightfold greater for poly dA than for poly rA (Fig. 2). Thus in terms of relative affinities for different polynucleotide lattices, the equilibrium and kinetic (dissociation) results appear to be in reasonable accord.

Conclusions The preliminary results cited above demonstrate that the dissociation kinetics of the GP32-nucleic acid lattice system are not compatible with either of the extreme mechanisms outlined above. Both the assumption of a diffusion-controlled association rate (see Lohman [6] for a further discussion of this aspect), and the assumption that sliding is either nonexistent or very fast, clearly require modification. Nevertheless, the data already available (in particular the dependence of k_d on fractional saturation and the absence of multiphasic dissociation) suggest strongly that isolated and contiguously-bound ligands interconvert during the dissociation process, rather than dissociating independently. Thus

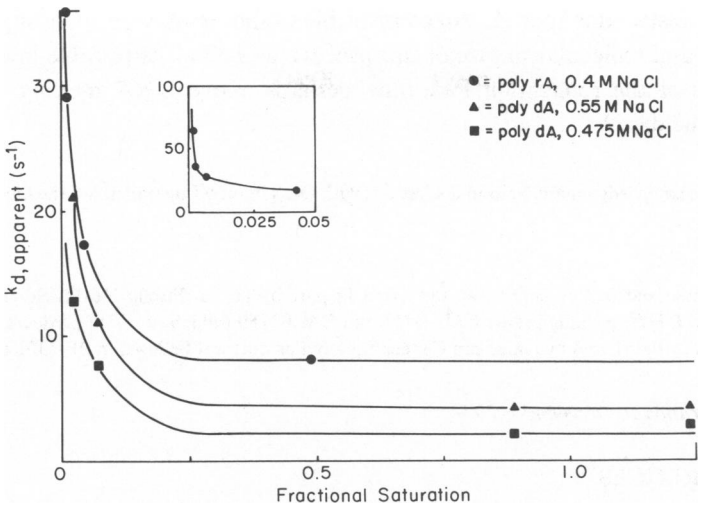

Figure 6 Plot of the dependence of $k_{d,\text{ apparent}}$ on the initial fractional saturation of the lattice. The experimental conditions are the same as those indicated in Fig. 7; the final salt concentrations used were 0.4–0.55 M NaCl (see figure).

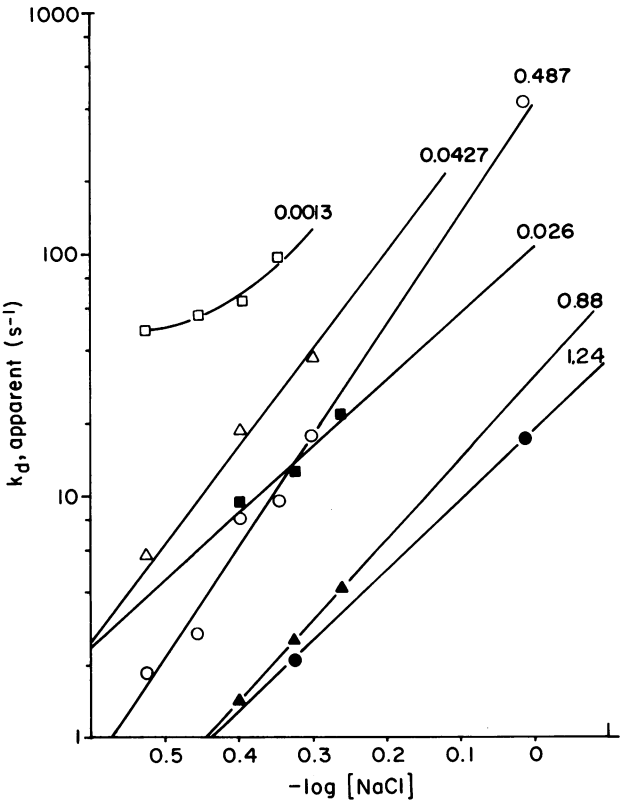

Figure 7 Plot of the log $K_{d,\text{ apparent}}$ versus log [NaCl] for poly rA (open symbols) and poly dA (filled symbols). The values of the initial ratio of protein to potential (nonoverlapping) binding sites are indicated adjacent to each line. The final concentration of GP32 in the poly rA experiments was 2.28×10^{-7} M, and in the poly dA experiments was 4.15×10^{-7} M; the buffer used was 10 mM Hepes, 0.1 mM EDTA, pH 7.7 plus added NaCl.

some protein translocation mechanism comparable to sliding may be operating in this system. The exact rate and molecular nature of this process, as well as its possible involvement in the overall kinetics of the replication fork (and perhaps native DNA melting with GP32* I), remain to be elucidated.

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DISCUSSION

Session Chairman: Alan Schechter *Scribe*: Donald W. Pettigrew

SCHECHTER: To initiate discussion, let me read a question from an anonymous referee. "In the absence of more definite kinetic information, especially on the association rate constants, I question the conclusion in your paper that 'some protein translocation mechanism comparable to sliding is clearly operating in this system.' There are many mechanisms which could give rise to single exponential decay kinetics, with a time constant that depends on the average degree of binding. For example, the rate limiting step could be a first order event which converts a whole polynucleotide (or some reasonable domain of the chain) to a rapidly dissociating form. The simple lattice model is an assumption. Demonstration of protein sliding will require a more direct experiment."

KOWALCZYKOWSKI: I agree. Although the model is correct, we made it very simple because there are as yet insufficient data to confirm the hypothesis. Since there is an intermediate operating, the situation is complex and needs further work. I don't want anyone to think that the form of dependence of the dissociation on binding density is direct support for a sliding model.

BLOOMFIELD: An observation made some years ago which made the sliding model attractive to me was that the apparent rate of combination of repressors with operator DNA was faster than diffusion controlled; Eigen and Richter proposed the sliding model to account for this. It is known from the work of Adam and Delbruck that reduction in dimensionality enables one to reach a given target much faster. I don't know whether those initial kinetics observations which, I think, were made in rather a crude way, have held up. If they have, they do provide some support for a sliding model.

KOWALCZYKOWSKI: I think that Mary Barkley would be more qualified to comment on experimental support for the model. Also, Bob Winter of Peter von Hippel's laboratory has found agreement of his data with theories developed by Otto Berg. So it does appear that *lac* repressor protein participates in one-dimensional diffusion along the lattice in search of its operator.

VON HIPPEL: Let me add a few provocative things to what Victor Bloomfield was saying. It is intriguing to think about sliding in this way. I held out against it as long as anybody because it just seemed awkward to me to think of a protein sliding along a nucleic acid surface, bumping up and down over the sugar-phosphate backbones. However, when one starts thinking about DNA as coated with condensed ions, then this concept becomes more attractive. In the terms outlined by Record et al., as the protein initially binds to the DNA a large entropic change occurs as the polycationic protein displaces a certain number of monovalent cations from the DNA. While the protein is sliding along you can imagine one ion going off and another coming on each time one base pair or residue is translated along the DNA. Thus it's not so difficult to imagine it sliding along what amounts to an isopotential surface. Whether this applies to the case Dr. Kowalczykowski is talking about remains to be seen. But at this level it is an attractive idea, especially if the other interactions with the backbone are not very specific. Others who want to think along those lines might find it a helpful view.

KALLENBACH: Sliding models seem to me to be intrinsically unlikely. Suppose we consider a person walking (randomly) near a sticky wall. What he gains in terms of dimensionality, the Adams-Delbruck effect, can come about simply by virtue of the fact that the wall effectively presents an enormous target once you leave it, one which you are highly likely to reencounter in a few steps.

So, the collision frequency becomes pseudo-2-dimensional simply by the fact that a rapid association-reassociation in the presence of a locally very high concentration of binding sites approximates what you would call sliding (in this case in 2-dimensions). It seems to me that you don't have to invoke sliding, despite Pete's idea of having a counter ion

on one side of the molecule suddenly release and jump on behind. You don't have to invoke any of that. Physically, the mechanisms become almost indistinguishable.

SCHECHTER: Can one formally distinguish them?

KALLENBACH: That's exactly the point. I don't see how you distinguish the gain, which is real in any case.

YAGIL: In discussing the question of sliding, if one thinks of the biological relevance of the problem, the dimension of packing of the DNA should not be forgotten. This packing is a well-established concept in eucaryotic nuclei, and is well known to occur also in procaryotic cells. Consequently, the problem of finding the target sequence is made much simpler. The possibility exists that only a minor fraction, and though perhaps an important minor fraction, is really accessible to regulatory molecules. It might be helpful to measure the rate at which repressor gets "on" and "off" non-specific DNA, organized either in nucleoid form (a la Warshel), or in a nucleosome form. Either the bending of the DNA, or its shielding by the nuclear protein, might reduce considerably the non-specific competition.

VON HIPPEL: It's complicated enough already—but you're right; this certainly needs to be considered in a complete description.

KOWALCZYKOWSKI: It's certainly true there are other proteins to look out for. The real question is how far things can move before bumping into each other. There is general agreement that a one-dimensional sliding event—a "hopping" or microscopic association/dissociation—would facilitate, even over a limited range, some processes which would be important biologically.

BARKLEY: I can see the rationale for sliding for site-specific interaction, but why would you want it for non-specific interactions?

KOWALCZYKOWSKI: It's actually quite important. Consider the case of cooperative binding of the protein in which you want to get to a certain level of saturation in a reasonable period of time. One of the major functions of the protein is to protect single-stranded DNA, which is transiently formed during replication or recombination, from nucleases. What you want to do is to achieve long contiguous clusters of the protein. So, let's say you want to make a cluster of length 10 proteins. If you say that the protein just randomly binds to a long piece of DNA, or to a gap, then the problem comes down to how to generate a cluster. For example, if one protein binds and the next one binds randomly, you might have a gap of let's say 4 nucleotides. The protein has a site size of 7 nucleotides, so it can't "fit" in a gap of size 4 nucleotides. Now, how do you decrease that gap of 4? You can dissociate one protein and bind again. But if it's at position 5 things are worse, so it comes back off and goes back on position 2 (i.e., one removed from the other bound protein). It's close, but its not quite there. So, sliding is certainly a means to facilitate contiguous binding even if we're not talking about sliding over large dimensions. If it hops a couple of bases, this would certainly allow more rapid, contiguous binding.

BARKLEY: Wait, you have another mechanism. Once you bind the protein, you can change the conformation of the DNA, or do something that's more recognizable than just a simple DNA sequence.

KOWALCZYKOWSKI: Yes, but if the limiting process is diffusion, i.e. if it's a diffusion-controlled reaction, it will work only if you can invoke some mechanism whereby the protein can know—at a distance—where it's going to go; but those distances are limited. If you're talking about binding to a large segment, and the protein's just binding randomly, it really won't know where it's supposed to bind until it gets to within a base pair or so. And when it's there, it binds cooperatively and it stays there because it has this extra stabilization of 4 kcal. And, if it's not there, it's unstable and dissociates. So, there is a constant search process for a cooperatively bound protein in order to completely saturate the lattice. In that sense, while not required for a non-specific non-cooperative protein, for something that's highly cooperative which does want to form large contiguous clusters, sliding could be an important mechanism.

KING: I think the problem is one of the passive word, "slide." These things are walking; they're not sliding. The cell is full of molecules that walk. All polymerases very, very actively walk down the molecule. It seems to me that those blocks are not passively sliding; they're probably actively walking to find havens.

SCHECHTER: There's no free energy involved during this mechanism.

KOWALCZYKOWSKI: No, we're assuming a random diffusion. I don't mean to suggest it's a greased-pole model of sliding. It could be walking, hopping — what have you.

KING: It's not a random diffusion. None of these molecules randomly diffuse on DNA. Polymerases do not randomly diffuse. They go one way.

KOWALCZYKOWSKI: Yes, but I think the difference between gene 32-protein and polymerases and things like ATPase-driven melting proteins, which *are* walking, is that the latter are actively driven by ATP, and this can provide directionality.

KING: These things can have directionality too, given that they have the cooperativity, which gives a reason for their finding each other. They're better off. You could still have a polar walk that wasn't ATP driven. And, since these things, as you say, have the function of covering that single-strand very efficiently, random walk is not the mechanism involved.

KOWALCZYKOWSKI: Yes, it would be difficult to distinguish between random diffusion and directional "walking". Nevertheless, both involve translocation along the DNA and both will accelerate the kinetic process.

KLAPPER: I get the image, from the way in which you describe this process, that you're thinking of putting on one subunit at a time, that subsequently finds its way down the chain to the right cluster. It seems to me that a diffusion-type reaction would involve all the molecules rapidly hopping on and off. When they find themselves clustered next to each other, they would stick more and the tendency to come off would go down. Hence, you would get a cooperativity in the rate constant as saturation was obtained. You wouldn't have to invoke any special sliding model since it's not necessarily any faster to have the protein slide about if it doesn't have to go to a specific site.

KOWALCZYKOWSKI: But in a certain sense they do have to go to a particular site: a site adjacent to protein that's already there. If you have a long polynucleotide lattice, and one protein molecule binds, the next one can bind anywhere it likes to. These things are taking up seven nucleotide spaces, and you now bring another one in, leaving a gap of two. The final equilibrium configuration is known, i.e., you know you have to have a certain distribution of clusters size 1, size 2, ... size 100. The question is, how do you get to a final cluster size of 100? If the process is being rate-limited by diffusion, is there a mechanism whereby they know to come straight out of solution and form clusters? To me that seems unlikely.

You can get final equilibrium by subsequent association and reassociation; there's no question about that. The potential for the sliding pathway exists and it would facilitate the rate of approach to equilibrium. The real question is whether the pathway does in fact exist for this protein system. It appears to act in the *lac* repressor system. One obvious question is whether this is a general phenomenon or is this something unique to the *lac* repressor.

VON HIPPEL: I wanted to provoke some discussion, and I seem to have succeeded. Neville Kallenbach's point may have gotten us off the track in the sense that this is a quantitative problem. Qualitatively, certainly, everything everybody's saying is true. Quantitatively, however, if you don't invoke some special mechanism, the on rate and the binding constant must be related directly to the off rate. You can then ask whether that's fast enough to get you where you want to go. These parameters are, to a first approximation, known. Lohman's and Kowalczykowski's work is still far from complete, so there may be intermediates whose nature we don't understand. The thing that distinguishes a facilitated movement problem such as sliding, or transfer (which is also a possible facilitating mechansim), is that you can avoid the activation problem. You can avoid having to rip yourself loose with what is required mathematically in terms of time. You can speed things up. So, having once examined the problem in a quantitative way, you can see that the chances are very high, mathematically as well as experimentally, that the simplest solution won't work. That's what we're trying to propose here. It's a question then of trying to find something that will work. This model comes close to fitting some of the data at present.

ACKERS: Apart from the interesting question of the kinetics involved, I think the thermodynamic relationships for cooperativity in systems of this kind offer some interesting comparisons. The problem is, how much cooperativity do you need to get sufficient coverage of the sites that have to be covered in order for a molecule to carry out its function? What you find, both experimentally and from theoretical interpretations, is that in order to cover many sites you need a cooperative energy of only ~4 kcal. I've analyzed data in collaboration with Mark Ptashne for the cooperative repressor binding to the right operator in the lambda system, in which one has to fill only two sites in order to get effective repression. In that case, the cooperative interaction energy between the repressors bound to adjacent sites is only ~2 kcal. That has the effect of covering both of the sites sufficiently so that one observes ~99.9% repression for transcription of early genes. If there are no cooperative interactions in that system, only ~95% repression would be observed at the concentration which corresponds to that of the lysogen repressor in the cell. So, that's enough cooperative energy to do the entire job. I think as more cases are studied, we'll find that there is an interesting

relationship between the magnitude of cooperative effects and the actual number and extent of coverage that one has to have.

KOWALCZYKOWSKI: As demonstrated by McGhee and von Hippel in their original paper, one of the problems for binding of a large ligand to a lattice is to achieve complete saturation, if that's required for its functioning. The problem is the severe entropic restriction in trying to pack the ligands in one next to the other. Under these conditions, a cooperativity parameter of 10^3 in their equations permits essentially complete (98–99%) saturation. If one of the proper functions of all single-stranded binding proteins is to protect single-stranded DNA, it is highly likely that they all will demonstrate some large amount of cooperativity, simply in order to achieve that saturation level.